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PRINCIPAL INVESTIGATOR: Ching-Juh Lai
AUTHORS: Chun-Fa Zhang, Michael Bray, Ruhe Men

CONTRACTING ORGANIZATION: National Institutes of Health
Molecular Viral Biology Section
Laboratory of Infectious Diseases
9000 Rockville Pike
Bethesda, Maryland 20892

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Ching-Juh Lai, Chun-Fa Zhang, Michael Bray, Ruhe Men

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Molecular Viral Biology Section
Laboratory of Infectious Diseases
9000 Rockville Pike
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Baculovirus recombinants that separately express the full-length E or NS1 glycoprotein of DEN4 or DEN2 or the full-length E of DEN3 were constructed. Recombinants expressing C-terminally truncated 80%E of the above three dengue serotype viruses were also constructed because earlier studies of recombinant vaccinia virus showed that this truncated 80%E is secreted extracellularly and is more immunogenic than the full-length E. The immunogenicity of baculovirus-expressed dengue virus E's was evaluated using the mouse dengue encephalitis model. Although the data for mouse protection would be more convincing were the mortality of negative control mice higher, sero-analysis provided evidence that the extracellular 80%E elicited higher antibody response than did the intracellular E or the full-length E. We were encouraged by this result and decided to test DEN4 80%E in a monkey experiment, to see if we could obtain a convincing protective response. The result showed that monkeys immunized with secreted 80%E developed low titers of neutralizing antibodies. Tests for post-challenge viremia failed to demonstrate viremia for reasons unclear at present. Analysis of sera indicated that all monkeys immunized with DEN4 E's were primed for a secondary antibody response to dengue virus challenge. This priming effect may be utilized in a two-phase vaccination strategy in which immunization with baculovirus-expressed E is followed by infection with a recombinant vaccinia virus or an under-attenuated live dengue vaccine in the second phase.

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INTRODUCTION

Dengue is a mosquito-borne viral disease that occurs in tropical and subtropical regions throughout the world. Dengue viruses (serotypes 1-4) are now members of the flavivirus genus within the newly classified flavivirus family (1). The dengue virus subgroup causes more human illnesses than any other member of the flavivirus genus. Dengue is characterized by fever, rash, severe headache, and joint pain. Its mortality rate is low. However, a more severe form of dengue, characterized by hemorrhage and shock (dengue hemorrhagic fever and dengue shock syndrome) has been observed with increasing frequency in children and young adults. Severe dengue occurs most often during dengue virus infection of individuals previously infected with another dengue virus serotype, and this has led to the suggestion that immune enhancement of viral replication plays a role in dengue pathogenesis (2-4).

Dengue virus contains a positive RNA genome and three structural proteins, i.e., the capsid protein (C), the small membrane protein (M), and the large membrane protein or envelope (E) glycoprotein. The RNA genome codes for a long polyprotein that is co- and post-translationally cleaved to produce the three structural proteins and a series of non-structural proteins designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in that order. The complete nucleotide and the deduced amino acid sequences of all four dengue virus serotypes including several strains of dengue type 2 virus have been obtained (5-10). This sequence information has been invaluable in our molecular studies to gain more insights into the biology, immunology, and pathogenesis of these viruses. Humans recovering from dengue illness, especially from secondary dengue virus infection, have high titers of antibodies to the dengue virus structural proteins, particularly the E glycoprotein, and to nonstructural protein NS1, in their sera (11). Evidence is available

indicating that E is a major protective antigen, including demonstration that antibodies to E neutralize dengue virus *in vitro* and immunization of mice with recombinant-expressed E induces resistance to dengue virus infection (12,13). NS1 is also important in inducing protective immunity, since mice immunized with the purified NS1 protein or recombinant vaccinia virus expressing NS1 are protected (14,15). Protection by NS1 antibodies is thought to involve immune recognition of NS1 on the cell surface, followed by complement dependent cytolysis.

One of our research goals has been to develop a safe and effective vaccine for prevention of dengue. The use of subunit proteins in immunoprophylaxis offers a number of theoretical advantages, but this has not been adequately explored. The baculovirus-insect cell system has been widely used to produce protein products of foreign genes, because: (1) a high level of expression of the foreign gene can be achieved, and (2) in a number of instances the insect cell system can process and modify expressed products to yield functionally and immunologically active proteins.

Earlier, we constructed a recombinant baculovirus that contained dengue type 4 virus (DEN4) cDNA that codes for the three structural proteins and nonstructural proteins NS1 and NS2A. Infection of cultured insect cells with this recombinant baculovirus produced what appeared to be authentic E and NS1 glycoproteins (16). Both protein products were glycosylated as indicated by their sensitivity to endo-glycosidase F digestion. Other encoded dengue virus proteins presumably were also produced. Immunization of mice with these dengue virus protein products induced solid resistance to lethal dengue virus challenge (16). This approach was extended by immunizing rhesus monkeys with lysates of recombinant baculovirus infected cells containing: (1) DEN4 C, precursor of M (PreM), E, NS1, and NS2A, or (2) DEN4 E alone (17). When these animals were challenged parenterally with a homotypic DEN4, only one of six

monkeys in the first group and only one of three monkeys in the second group were completely protected as indicated by the absence of viremia. Analysis of sera from immunized monkeys showed that the antibody response to E was very low or not detectable (17). Presumably for this reason, only partial resistance to intravenous dengue virus challenge was induced. Thus, the low immunogenicity of E is a major obstacle to the development of an effective dengue virus vaccine produced by recombinant DNA technology. Fortunately, our recent studies of recombinant vaccinia virus-expressed E showed that a C-terminally truncated E, approximately 80% in length, is expressed on the cell surface and is secreted in part extracellularly (18). The 80%E was more immunogenic and protective than the full-length E when tested in the murine dengue virus encephalitis model. Another advantageous property of 80%E is its secretion into the culture medium, thereby facilitating the isolation and purification of the E product. These properties of the engineered E glycoprotein should prove useful in immunoprophylaxis of dengue disease. It has become an important research goal to express the E glycoprotein in a more immunogenic form. As the first step toward this goal, we evaluated the immunogenicity and protective efficacy of these protein products in mice and in monkeys.

RESULTS

A. Recombinant Baculovirus Constructs Expressing Dengue Virus Proteins

Soon after the cloning and sequencing of dengue type 4 virus (DEN4) cDNA, we constructed a recombinant vaccinia virus that contained the 4.0 Kb Bgl II DEN4 cDNA fragment coding for the three structural proteins, (*i.e.*, C, PreM, and E), and nonstructural proteins NS1 and NS2A (19). Infection of CV-1 cells with this recombinant virus produced authentic DEN4 PreM, E, and NS1 glycoproteins. This successful effort prompted us to investigate the possibility of also expressing DEN4 glycoproteins in a baculovirus recombinant using the wild type Autographa californica mononuclear polyhedrosis virus (baculovirus) insect cell system. The same 4.0 Kb DEN4 cDNA fragment was inserted into baculovirus intermediate vector pAC373 provided by M. Summers (Texas A&M University, College Station, Texas) and a recombinant baculovirus designated b (DEN4, C-PreM-E-NS1-NS2A) was isolated using the standard method as described in the construction manual (16, 20). (The same procedure was followed for the construction of other recombinants using recently developed vectors such pVL941 or pBluBac). A series of baculovirus recombinants separately expressing DEN4 E or NS1 was prepared in an effort to clarify the role of E and NS1 in inducing resistance to dengue virus infection (21). For this purpose, we constructed recombinant baculovirus b (DEN4, 100%E) that codes for the predicted N-terminal hydrophobic signal sequence (amino acids 265-279) of E, the entire E sequence (amino acids 280-773) and N-terminal 4 amino acids of NS1 from the cloned DNA segment. Another recombinant, designated b (DEN4, 93%E), was constructed that codes for the N-terminal signal sequence, amino acids 1-456 lacking the C-terminal 39 hydrophobic amino acids of E. The C-terminal deletion was introduced, because it was thought that an E glycoprotein deleting

the putative C-terminal hydrophobic anchor could be secreted from the recombinant virus-infected cells, thus facilitating protein purification. A variant recombinant b (DEN4, RSVG-93%E) was also made that contained the hydrophobic signal sequence (amino acids 1-71) of the respiratory syncytial virus G glycoprotein (RSVG) replacing the putative DEN4 E signal (12). Subsequently, we showed that several C-terminally truncated E's, approximately 80% in length expressed by recombinant vaccinia virus, exhibited increased immunogenicity in mice. These truncated E's were detected on the cell surface and secreted extracellularly. Secreted E should be more desirable than the intracellular full-length E because of the ease of protein purification. The DEN4 cDNA sequences coding for the series of DEN4 80%E were readily available from the recombinant vaccinia virus constructs. For these reasons, we initiated studies to construct recombinant baculovirus for the production of 80%E truncated at the C-terminus.

We have shown earlier that proper processing of DEN4 NS1 requires the N-terminal hydrophobic signal and the downstream nonstructural protein NS2A (22). More recently, we also demonstrated that mice immunized with recombinant vaccinia virus expressing authentically cleaved NS1 are solidly protected against fatal dengue encephalitis (14). As described in the first annual report of this project, a recombinant baculovirus that contained DEN4 cDNA coding for NS1-NS2A was constructed and isolated. This recombinant baculovirus was designated b (DEN4, NS1-NS2A). Dr. H. Hori in the laboratory showed that recombinant vaccinia virus v (DEN4, NS1) containing DEN4 cDNA that codes for the N-terminal signal and the entire length of NS1 produced 2-3 fold more NS1 glycoprotein than did recombinant v (DEN4. NS1-NS2A). To achieve such high level expression of NS1, it was desirable to construct a recombinant baculovirus expressing NS1 in the absence of NS2A thus requiring no additional processing step.

This recombinant was constructed using intermediate vector pVL941 and was designated b (DEN4, NS1).

The dengue virus complex of the flavivirus genus consists of 4 serotypes, all of which cause dengue epidemics in tropic areas. The current concept for immunization against dengue favors the use of a vaccine preparation that contains antigens of all four serotypes. There is a high degree of conservation of the amino acid sequences among the four dengue virus serotypes: amino acid homology of E varied from 62-70% and amino acid homology of the NS1 sequences varied from 66-73%. The same construction strategy of DEN4 recombinants was adapted to construct recombinant baculovirus expressing DEN2 full-length (100%) E, C-terminally truncated 80% E, or NS1-NS2A using DEN2 S1 strain derived cDNA clones kindly provided by J. Strauss (California Institute of Technology, Pasadena, CA) through R. Patnak (WRAIR, Washington, DC). Recombinant baculovirus b (DEN3, 100%E), or b (DEN3, 80%E), expressing the full-length E or the highly immunogenic 80%E, respectively, were also constructed using DEN3 cDNA clones of strain H87 kindly provided by H. Sumiyoshi (CDC, Fort Collins, CO). Thus far, a total of 12 baculovirus recombinants expressing the protective antigens of 3 dengue virus serotypes was constructed (see Table 1). DEN1 cDNA sequences were not available in our laboratory at that time. Since DEN1 DNA sequences are now available elsewhere and in our laboratory as well, those DEN1 baculovirus recombinants proposed earlier can be readily constructed as needed.

B. Analysis of Dengue Virus Proteins in Baculovirus-Infected Cells

(1) Detection of Dengue Virus E Glycoproteins. All recombinant baculoviruses were selected and purified from the wild type baculovirus by serial-dilution passage and visual

selection of virus plaques lacking polyhedrin. Initial positive identification of recombinant baculovirus was performed by an indirect immunofluorescence assay (IFA) using dengue hyperimmune mouse ascitic fluid (HMAF). To verify the expressed protein product, insect sf9 cells were infected with the recombinant baculovirus, labeled with ^{35}S-methionine , and lysed in RIPA buffer (1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl). The radio-labeled lysate prepared in this manner was used for immunoprecipitation followed by analysis on SDS polyacrylamide gel electrophoresis (PAGE). Recombinants b (DEN4, 93%E) and b (DEN3, 100%E) were readily shown to produce an E glycoprotein of expected size. However, although cells infected with b (DEN4, 100%E) or b (DEN2, 100%E) were strongly positive by IFA, the products of these recombinants were not detected by radio-immunoprecipitation (RIP) and SDS-PAGE. It is thought that this may be the result of strong binding of these proteins to lipid membranes of sf9 cells, mediated by the hydrophobic C-terminal domain.

(2) Detection of Dengue Virus NS1. To verify the expression of DEN4 NS1, the lysate of sf9 cells infected with b (DEN4, NS1-NS2A) or b (DEN4, NS1) was immunoprecipitated and analyzed by SDS-PAGE. The result showed that a protein approximately 40-44 Kd of the size predicted for DEN4 NS1 was detected. The estimated molecular size suggested that the NS1 product was glycosylated. Similarly, analysis of DEN2 NS1 produced by recombinant b (DEN2, NS1-NS2A) was also performed. The result also showed that a protein band of size approximately 40-44 Kd, indicative of a glycosylated NS1, was made in recombinant virus-infected cells.

It has been reported that a small fraction of dengue virus NS1 is secreted into the medium of dengue virus-infected cells (23). We also showed that extracellular DEN4 NS1 was detected

at a low level (<2%) during infection of CV-1 cells with a recombinant vaccinia virus (B. Falgout and C.-J. Lai, unpublished data). Extracellularly secreted NS1 migrated slower than did intracellular NS1 indicating that additional glycosylation had occurred during its transfer through the exocytic pathway. As part of our analysis of DEN4 or DEN2 NS1 produced by recombinant baculovirus, the medium fraction of ^{35}S-methionine labeled infected cells was also immunoprecipitated and separated by SDS-PAGE. The result indicated that extracellular NS1 was not detected.

C. Synthesis and Secretion of Dengue Virus E Glycoproteins

Synthesis and secretion of baculovirus-expressed DEN4 80%E product was analyzed in a pulse-chase experiment. Detection by RIP and SDS-PAGE (Fig. 1) revealed that a glycoprotein of expected size was found in the lysate of cells infected with b (DEN4, 80%E). After 2-hour labeling and a 6-hour chase, the concentration of the 46-50 Kd DEN4 80%E protein in the medium was approximately 15% of that present in the infected cells. Examination of the medium (Fig. 1, lane 6) revealed that a protein band co-migrated with the labeled 80%E protein, but the majority of this material failed to immunoprecipitate with HMAF, and proved to be the baculovirus storage protein. Secreted DEN4 80%E would have to be purified to remove this and other viral or insect cell proteins before it could be used for human immunization. However, for animal immunization, the secreted 80%E protein can be readily concentrated by centrifugation of the medium through a Centricon.

Analysis of the product of b (DEN2, 80%E) showed it to be similar to that of its DEN4 counterpart (Fig. 2). Since the DEN2 100%E product in the infected cell lysate could not be detected by SDS-PAGE, the amount of E used for immunization was quantitated by an indirect

method using the same dilution or infection with the same moi and for the same length of time as b (DEN2, 80%E).

Analysis of the DEN3 E protein in the lysate of recombinant virus-infected cells was also performed. The result showed that cells infected with recombinant b (DEN3, 100%E) and b (DEN4, 80%E) produced E proteins of expected size (data shown in last year's annual report). Although the medium of cells infected with b (DEN3, 100%E) showed a low level of what appeared to be 100%E protein, this probably represented leakage of labeled E as a result of cell lysis at a late stage of infection. The level of secreted 80%E was somewhat lower, relative to the intracellular protein than was observed for the DEN4, or DEN2 80%E shown above.

D. Immunization of Mice with Dengue Virus E Glycoproteins: Protection Against Fatal Dengue Encephalitis

(1) DEN4 E Glycoproteins. The murine dengue virus encephalitis model was employed to evaluate the protective efficacy and immunogenicity of dengue virus E glycoproteins produced by recombinant baculovirus. The relative immunogenicity of the intracellular or extracellular DEN4 E was determined by inoculating identical quantities into mice and assessing the antibody response. The concentrations of intracellular DEN4 93%E and 80%E in cell lysates and extracellular DEN4 80%E in concentrated medium were adjusted so that mice received same quantity of antigen. Sero-analysis (Fig. 3) showed that all mice inoculated with secreted 80%E (lanes 8--14) and one of three mice received the lysate of b (DEN4, 93%E) infected cells (lane 4) developed antibodies to E, while other mice inoculated with a lysate of cells infected with b (DEN4, 93%E), or b(DEN4, 80%E) showed a low antibody response as determined by RIP. Quantitation of neutralizing antibodies in mouse sera was not performed.

Table 2 shows the results of two experiments to determine the protective efficacy of the baculovirus-expressed DEN4 E products in mice. Although the negative control groups immunized with a lysate of wild type baculovirus infected cells showed less than 50% mortality, the combined mortality of these groups was still significantly higher than the combined mortality of the groups inoculated with the product of b (DEN4, 93%E), or with the intracellular or secreted product of b (DEN4, 80%E) ($p < .025$). In terms of morbidity, all mice immunized with the wild type product developed signs of encephalitis following DEN4 challenge, while none immunized with the secreted 80%E product showed signs of disease. Approximately half of all mice given the 93%E product and nearly all mice given the intercellular 80%E developed encephalitis.

(2) DEN2 E Glycoproteins. The immunogenicity of baculovirus-expressed DEN2 100%E and 80%E was evaluated as described in Fig. 4. All mice inoculated with the extracellularly secreted 80%E developed a strong antibody response, while the antibody response of mice given the intracellular 80%E was weaker. Mice given the 100%E product or wild type baculovirus infected cell lysate did not develop detectable antibodies to E. The antibody response to secreted E was comparable to that seen in mice immunized with recombinant vaccinia virus expressing DEN2 80%E. The protective efficacy of these DEN2 E products in mice was also determined (Table 3). The secreted form of DEN2 80%E induced a significant level of protection compared to the mortality of negative control mice ($p<.025$).

(3) DEN3 E Glycoproteins. Mice were inoculated with lysates of cells infected with b (DEN3, 100%E), b (DEN3, 80%E), or with concentrated supernatant from cells infected with b (DEN3,80%E), As described in last year's report, only mice inoculated with secreted 80%E developed detectable antibodies against the authentic DEN3 E protein. Sera from immunized

mice were further tested for the presence of protective antibodies in a passive transfer experiment. Suckling mice were inoculated intraperitoneal with the immune serum, followed by intracerebral challenge with mouse-brain adapted DEN3 (strain H87). Transfer of serum into suckling mice had to be performed because the DEN3 strain has not been successfully adapted to growth in mouse brain to the point that it will cause fatal encephalitis in adult mice. Studies were designed in which the protective efficacy of baculovirus DEN3 E products was evaluated. Both experiments, shown in Table 4, failed to show protection by experimental immune serum, though DEN3 HMAF was protective in one of the experiments. It is possible that the level of E antibodies in sera of immunized mice was low and not sufficient to provide resistance to fatal DEN3 challenge.

E. Immunization of Rhesus Monkeys with DEN4 E Glycoproteins

The results obtained thus far established that dengue virus 80%E glycoprotein expressed by recombinant baculovirus b (DEN4, 80%E), b (DEN2, 80%E), or b (DEN3, 80%E) is secreted into the medium fluid. This unique property of dengue virus E was also reported by another research group working with another strain of dengue type 2 virus (24). We showed that mice inoculated with the secreted DEN4 80% E in concentrated medium developed a stronger antibody response to DEN4 E than did mice immunized with the lysate of cells infected with b (DEN4, 80%E), b (DEN4, 93%E) constructed for the present analysis, or with b (DEN4, C-Pre-M-E-NS1-NS2A), or b (DEN4, RSVG-93%E) previously derived. Since the DEN4 protein products of the latter two recombinants appeared to partially protect rhesus monkeys against challenge with wild type homotypic DEN4 in an earlier experiment, we decided to test the product of b (DEN4,

80%E) in a second monkey experiment to determine if we could obtain a more convincing protective response.

(1) Immunization. Lysates were prepared from insect sf9 cells infected with b (DEN4, 80%E), b (DEN4, 93%E), or the wild type baculovirus at an moi of 1-2 pfu for 72 hr. In general, $2X10^6$ infected cells in a T_{150} flask were scraped into 1 ml phosphate buffered saline (PBS), dounced, and the homogenate (cell lysate) was used for immunization. To prepare extracellular 80%E, growth medium was removed from infected cells 36 hr after infection and replaced with fresh growth medium containing no added serum. The medium fraction (12 ml/flask) was collected 15-24 hr later and concentrated by centrifugation in a Centricon tube to approximately 0.8 ml/flask.

Young rhesus monkeys in groups of four were inoculated intramuscularly and subcutaneously with: (A) a lysate of cells infected with b (DEN4, 93%E), (B) a lysate of cells infected with b (DEN4, 80%E), or (C) concentrated medium from cells infected with b (DEN4, 80%E). As controls, two monkeys received a lysate of cells infected with the wild type baculovirus and another two received concentrated medium from the same cells. The experimental protocol is shown in Fig. 5. Each monkey was inoculated three times with 2 ml of the cell homogenate or the equivalent amount of E in concentrated medium as measured by Western blotting. Booster immunization was done at 4 weeks and again at 15 weeks. All monkeys were challenged at 18 weeks by subcutaneous injection of 10^5 pfu/dose of DEN4 strain 341750 in a lyophilized preparation (1/86) kindly provided by D. Dubois and K. Eckels (WRAIR, Washington, DC). Monkeys were bled during the course of immunization to evaluate their immune response and after challenge to assay for viremia.

(2) Analysis of Antibody Response by RIP. Sera from monkeys immunized with DEN4 E glycoproteins were analyzed by RIP using ^{35}S-methionine labeled proteins of DEN4 infected $LLCMK_2$ or C6/36 cells. The labeled immunoprecipitates were separated on SDS-PAGE. The result (Fig. 6) shows that each of the four monkeys immunized with concentrated medium containing secreted 80%E developed E antibodies following the initial booster immunization (lanes 2). The level of antibodies increased further after the second booster immunization (lanes 3) as judged by the intensity of the precipitated E band. Two of four monkeys immunized with the cell lysate containing intracellular 80%E developed low to moderate E antibody response. The level of antibody response did not appreciably increase after the second booster immunization. Monkeys immunized with a lysate containing intracellular 93%E developed little, if any, E antibodies detected by RIP throughout the course of immunization.

(3) Analysis of Antibody Response by Plaque Reduction Neutralization Test. Antibody response to immunization was also analyzed by 50% plaque reduction neutralization test (25). Analysis of pre-challenge sera showed that 3 monkeys immunized with extracellular DEN4 80%E had a titer of 1:20, and another animal in the group had a titer of 1:10. Two of four monkeys which received b (DEN4, 80%E) infected lysate had a titer of 1:10, and one of four monkeys in the group which received a lysate containing intracellular 93%E had a titer of 1:10. All other monkeys including controls were negative (< 1:10) by this assay. Overall, the result showed that the extracellular form of DEN4 80%E was slightly more immunogenic than its intracellular counterpart. The lysate containing intracellular DEN4 93%E was least immunogenic. However, none of the preparations exhibited satisfactory immunogenicity.

(4) Evaluation of the Protective Efficacy Against Dengue Virus Challenge. The response of rhesus monkeys to dengue virus infection is similar to that in humans in that there are 4-6

days of viremia. Infection of monkeys represents the closest experimental analogue of human dengue virus infection. To detect DEN4 in the blood of inoculated monkeys, serum samples were used to inoculate C6/36 cells and the medium fluid was harvested 14 days later. Amplified virus was detected by plague assay on C6/36 cells. Control monkeys immunized with the wild type baculovirus materials were positive on a few scattered days. None of the monkeys sustained four to six days of viremia. Monkeys immunized with DEN4 93%E or 80%E also exhibited viremia on scattered days. The cause of this failure to induce resistance is not known. Because of the failure of control monkeys to develop full-blown viremia, a definite conclusion can not be drawn from this experiment as to the protective efficacy of these baculovirus products in monkeys. But, it is evident that these DEN4 E glycoproteins were only weakly immunogenic in primates.

(5) Sero-Response Following Dengue Virus Challenge. Monkeys were bled for analysis of antibody response at 2, 3, 4, 6, and 8 weeks following dengue virus challenge. The results in Fig. 7 show that at 2 weeks, all 12 monkeys immunized with DEN4 E glycoproteins developed an enhanced E antibody response to challenge, while little or no E antibody response was detected in control monkeys. Enhanced E antibodies that developed in monkeys following challenge represent an accelerated secondary response primed by immunization with baculovirus expressed E. Such an antibody response to E was not sustained, because control monkeys attained a similar high level of E antibodies at 6 weeks following challenge. At this time, antibody response to NS1 was also evident. It is interesting to note that two of four monkeys immunized with extracellular 80%E and two of four immunized with intracellular 80%E developed low level of NS1 antibodies. NS1 antibodies were also low in one of the monkeys immunized with 93%E and in one of the control monkeys. Since the results of the viremia

assay were not conclusive, it could not be established whether those monkeys which developed a low level of NS1 antibodies were partially protected. It remains to be determined whether a priming effect can be utilized in a two-phase immunization scheme in which priming with subunit proteins expressed by baculovirus recombinants in the first phase is followed by infection with a recombinant vaccinia virus or an under-attenuated live dengue vaccine in the second phase.

CONCLUSIONS

The dengue virus envelope (E) glycoprotein and nonstructural NS1 glycoprotein have been identified as independent protective antigens in mice during studies in which these glycoproteins expressed singly or together by a recombinant vaccinia virus were evaluated for their protective immunity against lethal dengue encephalitis. The use of these glycoproteins in a subunit form in dengue immunoprophylaxis offers a number of advantages, but this has not been adequately explored. As proposed, the major goals of the present study are: (1) to produce the authentic form of these subunit protective antigens, and (2) to engineer recombinant DNA that codes for highly immunogenic protein products using the high yielding baculovirus-insect cell expression system.

In the first phase of this study, we constructed a series of baculovirus recombinants that separately expressed full-length E or NS1 glycoprotein of dengue type 4 or type 2 virus, or the full-length E of dengue type 3 virus. We also constructed baculovirus recombinants for the production of C-terminally truncated 80%E of dengue type 2, 3, or 4 virus. These recombinants expressing re-engineered E's were constructed because of our recent finding that a C-terminally truncated DEN4 E, approximately 80% in length, exhibited increased immunogenicity in mice. In addition to being more immunogenic, a significant fraction of the E product was secreted extracellularly. A total of 12 baculovirus recombinants including several that produced NS1 glycoprotein were made. Radio-immunoprecipitation was performed to analyze the dengue virus product in the lysate of recombinant virus infected cells. The results showed that insect cells infected with recombinant b (DEN4, 93%E), b (DEN3, 100%E), b (DEN4, 80%E), b (DEN2, 80%E), or b (DEN3, 80%E) produced a protein of the molecular weight predicted for the

encoded glycoprotein. Similarly, recombinant b (DEN4, NS1) or b(DEN2, NS1) produced a protein in infected insect cells that was identified as NS1 glycoprotein. The product of b (DEN4, 100%E) or b (DEN2, 100%E) was not detected by this analysis, presumably because there was strong binding of these proteins to lipid membranes of the host cell.

In the second phase of this study, the immunogenicity and protective efficacy of the dengue virus E glycoprotein products were evaluated using the mouse dengue encephalitis model. The data for mouse protection against homotypic dengue virus challenge might have been more convincing had the mortality of control mice been higher. The result of sero-analysis by RIP provided evidence that the secreted 80%E of each of the three dengue virus serotypes tested elicited a higher antibody response than did their respective intracellular counterpart or the full-length E protein. We were encouraged by this finding because DEN4 E products that were shown to be poorly immunogenic and were partially protective in an earlier primate study. We decided to test DEN4 80%E in a second monkey experiment, to determine if we could observe a more convincing protective response.

The results of the study showed that antibody response to immunization was low as determined by plaque reduction neutralization test. Two of four monkeys which received 80%E secreted into the medium had a titer of 1:20. One of four monkeys which received 93%E lysate and two of four which received intracellular lysate of 80%E had a titer of 1:10. Other monkeys, including controls, were negative (<1:10). Tests for post-challenge viremia, using amplification on C6/36 cells followed by plague assay on the same cells failed to demonstrate viremia; the cause of this failure is not known. Analysis of seroresponse following dengue virus challenge indicated that all monkeys immunized with the DEN4 glycoprotein products developed an enhanced E antibody response to challenge. This represents an accelerated secondary response

primed by immunization. As suggested earlier, such a priming effect may be utilized in a two phase immunization scheme in which priming with subunit proteins expressed by baculovirus recombinants in the first phase is followed by infection with a recombinant vaccinia virus or an under-attenuated live dengue vaccine in the second phase.

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TABLE 1. Baculovirus recombinants that include dengue virus cDNA constructed for evaluation of immunogenicity and protective efficacy of the expressed dengue virus protein(s).

Baculovirus Recombinants	Dengue Virus Protein(s) Expressed (Amino Acid No.)[c]	Baculovirus Expression Vector
b(DEN4, C-M-E-NS-NS2A)	1–1344	pAC373
b(DEN4, 100%E)	280–735	pAC373
b(DEN4, RSVG-93%E)[a]	265–777	pVL941
b(DEN4, 93%E)	265–735	pVL941
b(DEN4, 80%E)	265–673	pVL941
b(DEN4, NS1-NS2A)	749–1344	pVL373
b(DEN4, NS1)	749–1126	pVL941
b(DEN2, 100%E)	265–747	pVL941
b(DEN2, 80%E)	265–676	pVL941
b(DEN2, NS1-NS2A)[b]	737–1467	pVL941
b(DEN3, 100%E)	266–773	pBlueBac
b(DEN3, 80%E)	266–675	pBlueBac

[a]The N-terminal sequence (amino acids 1-71) of respiratory syncytial virus G glycoprotein (RSVG) which contains the hydrophobic membrane anchor was employed as a signal for proper processing and glycoprotein of E.

[b]The dengue virus cDNA insect also codes for additional sequences including the last 39 amino acids of E and the N-terminal 122 amino acids of NS2B.

[c]The amino acid numbers refer to the positions of the respective polyprotein sequence published for DEN4 (5, 6), DEN2 (7,, 8, 8a), and DEN3 (10).

TABLE 2.

Response to dengue 4 virus i.c. challenge of mice immunized with recombinant baculovirus-expressed dengue 4 envelope glycoprotein products

Product expressed by recombinant baculovirus	Experiment 1 Mortality	Experiment 2 Mortality	Combined Morbidity
DEN4 93% E lysate	0/10	0/10	40-50%
DEN4 80% E lysate	0/10	1/10	90%
DEN4 80% E supernatant	0/10	0/10	0
WT baculo lysate	3/10	4/9	100%

Female BALB/c mice were inoculated i.m. at age 3 weeks with a total of 0.1 ml lysate or supernatant preparation, boosted 2 weeks later, and challenged at age 6 weeks by intra-cranial inoculation of 100 LD_{50} of mouse-brain-adapted H241 strain dengue 4 virus. The mice were observed for 28 days for signs of encephalitis and for death.

TABLE 3.

Response to dengue 2 virus i.c. challenge of mice immunized with recombinant baculovirus-expressed dengue 2 envelope glycoprotein products

Product expressed by recombinant baculovirus	Morbidity	Mortality
DEN2 100% E lysate	12/12	9/12
DEN2 80% E lysate	12/12	5/12
DEN2 80% E supernatant	5/12	3/12
WT baculo lysate	14/14	10/14

Female BALB/c mice were inoculated i.m. at age 3 weeks with a total of 0.1 ml lysate or supernatant preparation, boosted 2 weeks later, and challenged at age 6 weeks by intra-cranial inoculation of 100 LD_{50} of mouse-brain-adapted New Guinea C strain dengue 2 virus. The mice were observed for 28 days for signs of encephalitis and for death.

TABLE 4.

Response to dengue 3 virus challenge of suckling mice after inoculation with serum from mice immunized with recombinant baculovirus-expressed dengue 3 envelope glycoprotein products

Product expressed by recombinant baculovirus	Experiment 1 Mortality	Experiment Mortality
DEN3 100% E lysate	9/9	9/11
DEN3 80% E lysate	9/9	9/11
DEN3 80% E supernatant	10/10	9/11
WT baculo lysate	12/12	8/11
POSITIVE CONTROLS:		
anti-DEN3 immune serum (HMAF) 1:10 dilution		0/7
"" 1:100	6/6	11/11

Serum donors: female BALB/c mice were inoculated i.m. at age 6 weeks with a total of 0.2 ml lysate or supernatant preparation and boosted 2 weeks later. Beginning one week after boosting the donors were bled 4 times, at 5-7 day intervals, and the serum from each group was pooled. Positive control HMAF was provided by WRAIR. Suckling BALB/c mice (4 days old in Experiment 1, 6 days old in Experiment 2) were inoculated intra-peritoneally with .050 ml (Experiment 1) or .10 ml (Experiment 2) of serum, and challenged the following day by intra-cranial inoculation of 100 LD_{50} of mouse-brain-adapted H87 strain dengue 3 virus. The mice were observed for 23 days for signs of encephalitis and for death.

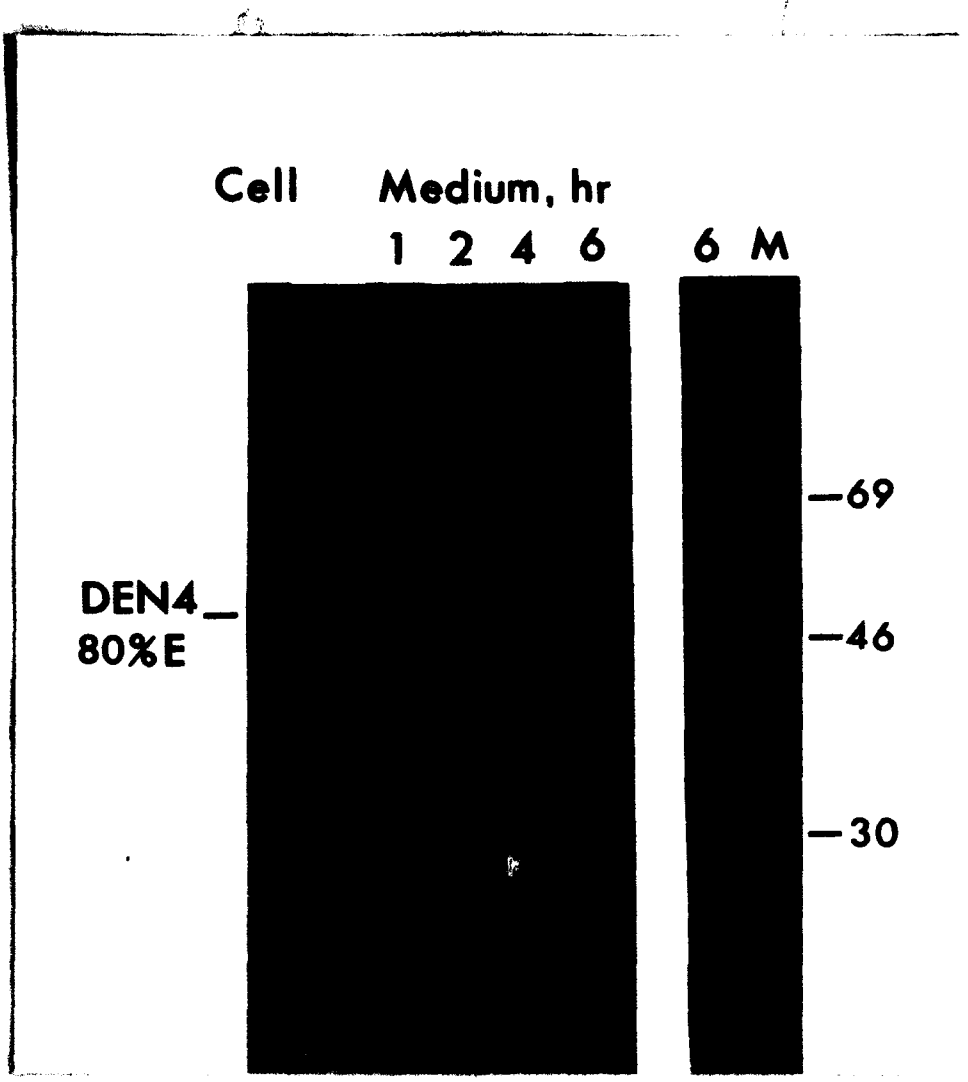


FIGURE 1. Secretion of dengue type 4 virus 80% envelope (E) protein from Sf9 cells infected with recombinant baculovirus

Confluent S. frugiperda Sf9 cells infected with recombinant baculovirus were radio-labeled for 2 hours with ^{35}S-methionine, and the labeling medium was replaced with serum-free Grace's medium. Aliquots of medium were collected at the indicated times. At 6 hours the medium was removed and the cells lysed. Equivalent aliquots of medium and cell lysate were immunoprecipitated with anti-dengue 4 HMAF, followed by SDS-12% PAGE, to detect extracellular and intracellular 80% E. The 6-hour medium sample was also analyzed without immunoprecipitation (column at right). M: molecular weight markers in kilodaltons.

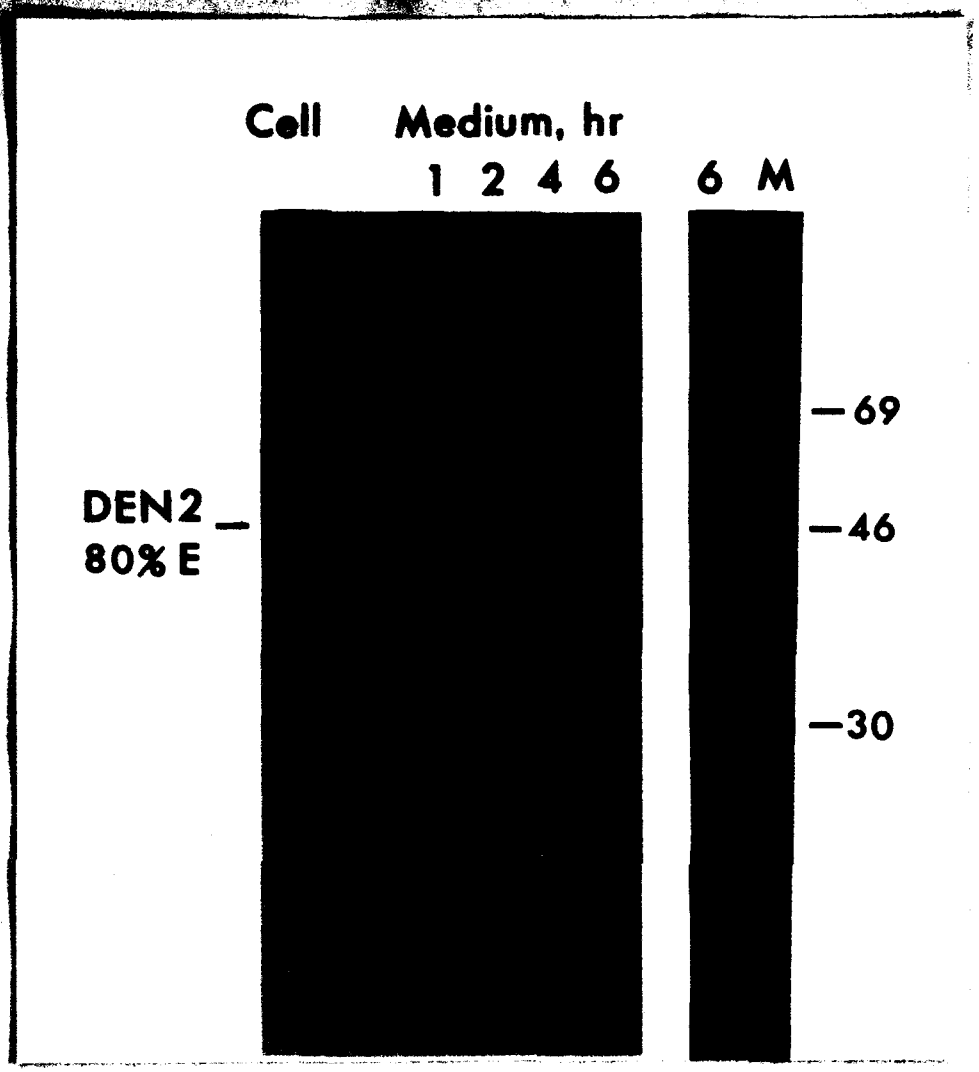
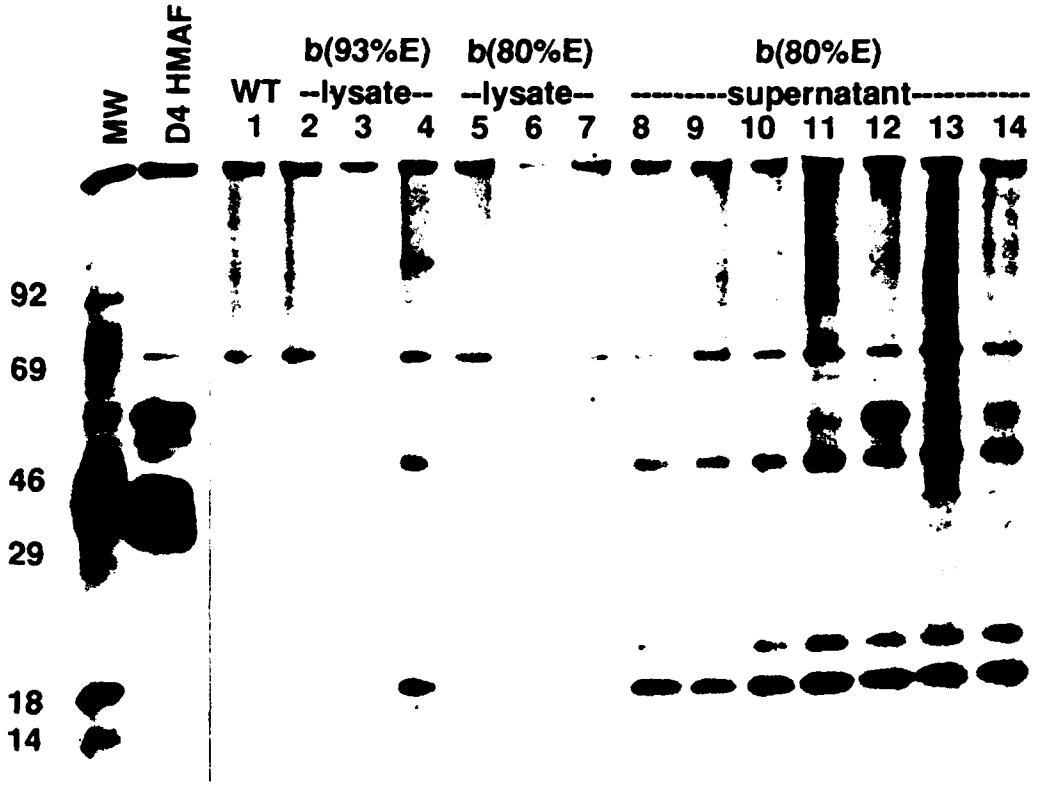


FIGURE 2. Secretion of dengue type 2 virus 80% E from Sf9 cells infected with recombinant baculovirus.

See the legend to Figure 1 for details.

FIGURE 3.

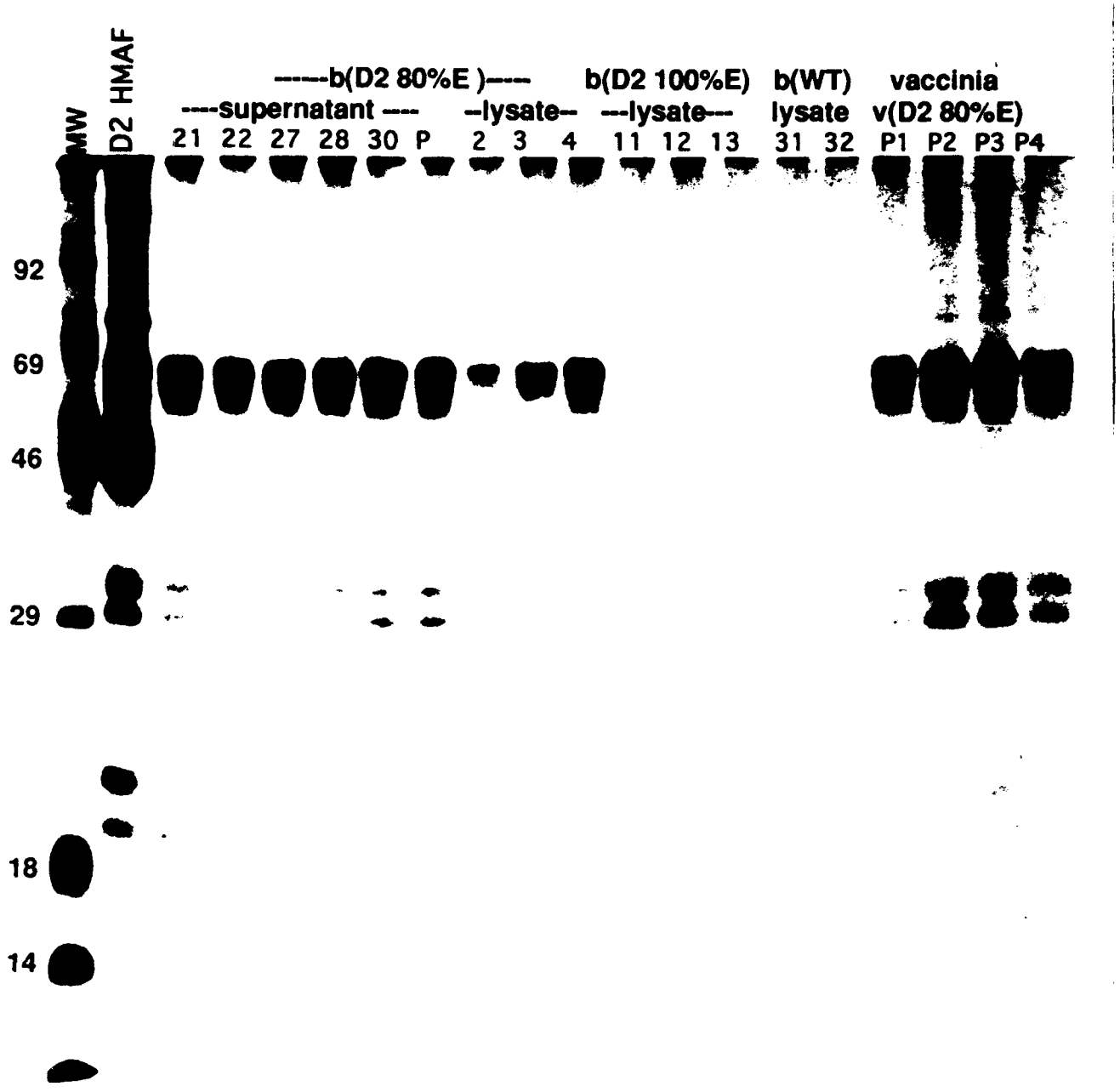
Seroresponse of mice to dengue type 4 envelope (E) glycoproteins produced by cells infected with recombinant baculovirus



Three-week-old female BALB/c mice were immunized i.m. with a total of 0.1 ml of material containing an identical amount of dengue 4 E product, as determined by Western blotting and probing with antiserum and ^{125}I-labeled staph protein A, or with a diluted lysate of cells infected with wild-type baculovirus. The mice were boosted two weeks later, then bled one week after boosting. Sera were used to immunoprecipitate a lysate of radio-labeled dengue 4 -infected cells, followed by SDS-12%PAGE.

FIGURE 4.

Seroresponse of mice immunized with dengue type 2 100% or 80% envelope (E) proteins produced by recombinant baculovirus or with 80% E produced by recombinant vaccinia virus



A. Immunization with baculovirus-expressed products: Three-week-old female BALB/c mice were immunized i.m. with 0.1 ml of material containing an identical amount of D2 80% E , either in an Sf9 cell lysate or concentrated cell supernatant, as determined by Western blotting and probing with dengue 3-specific antiserum and ^{125}I-labeled staph A protein, or with a lysate of cells infected with b(DEN2, 100%E) at the same M.O.I., for the same length of time, as for b(DEN2, 80%E), and diluted to the same extent, or with a lysate of cells infected with wild-type baculovirus at the same M.O.I., diluted to the same extent. They were boosted 2 weeks later, and bled at age 6 weeks.
B. Immunization with recombinant vaccinia virus expressing dengue type 2 80% E The same schedule was followed with vaccinia immunization; each mouse was inoculated i.p. with 3×10^6 p.f.u. of virus; the mice were bled four times, beginning at age 6 weeks, and the serum pooled.

Fig. 5 Monkey Immunization and Challenge Protocol

Week 0 – Immunize monkeys with baculovirus expressed E (IM and SC)

Week 4 – Immunize monkeys with baculovirus expressed E (booster)

Week 15 – Immunize monkeys with baculovirus expressed E (booster)

Week 18 – Challenge monkeys with dengue type 4 virus at $10^{5.0}$ pfu/dose (SC)

Week 18 & Days 1-13 Post-Challenge – Bleed monkeys for viremia assay

Weeks 4, 6, 8, 18, & 20 – Bleed monkeys for antibody response analysis

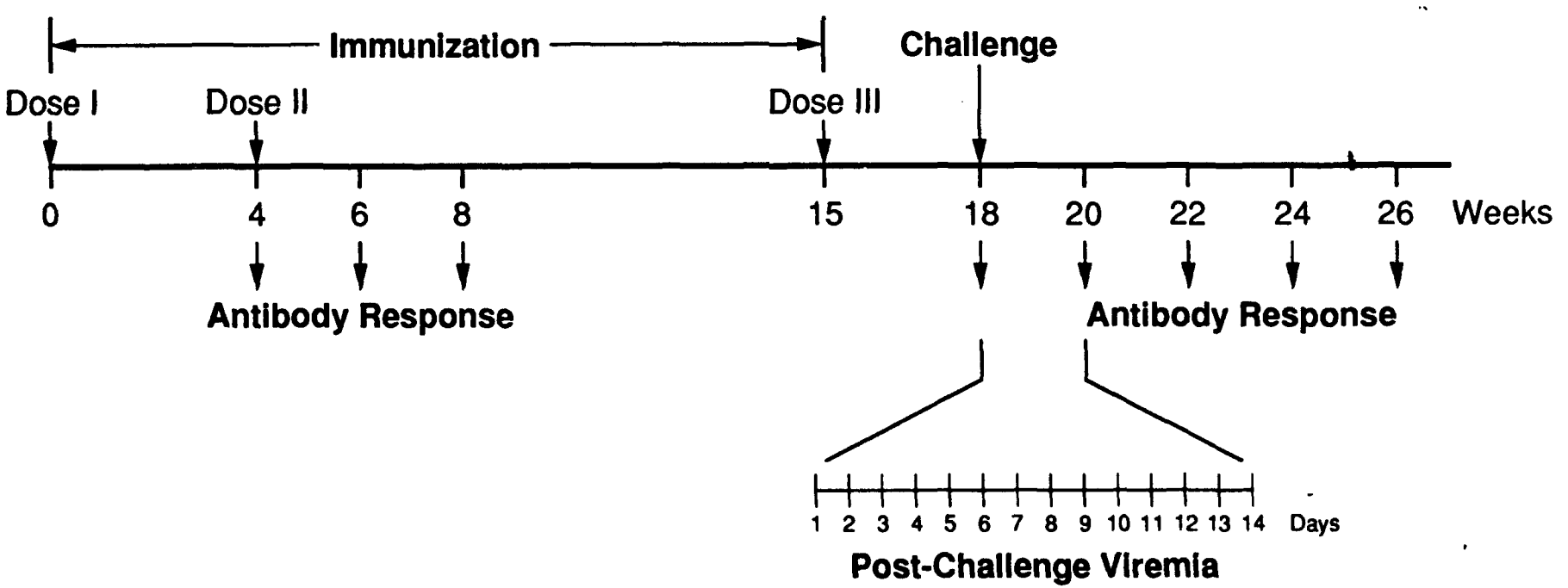


Fig. 6

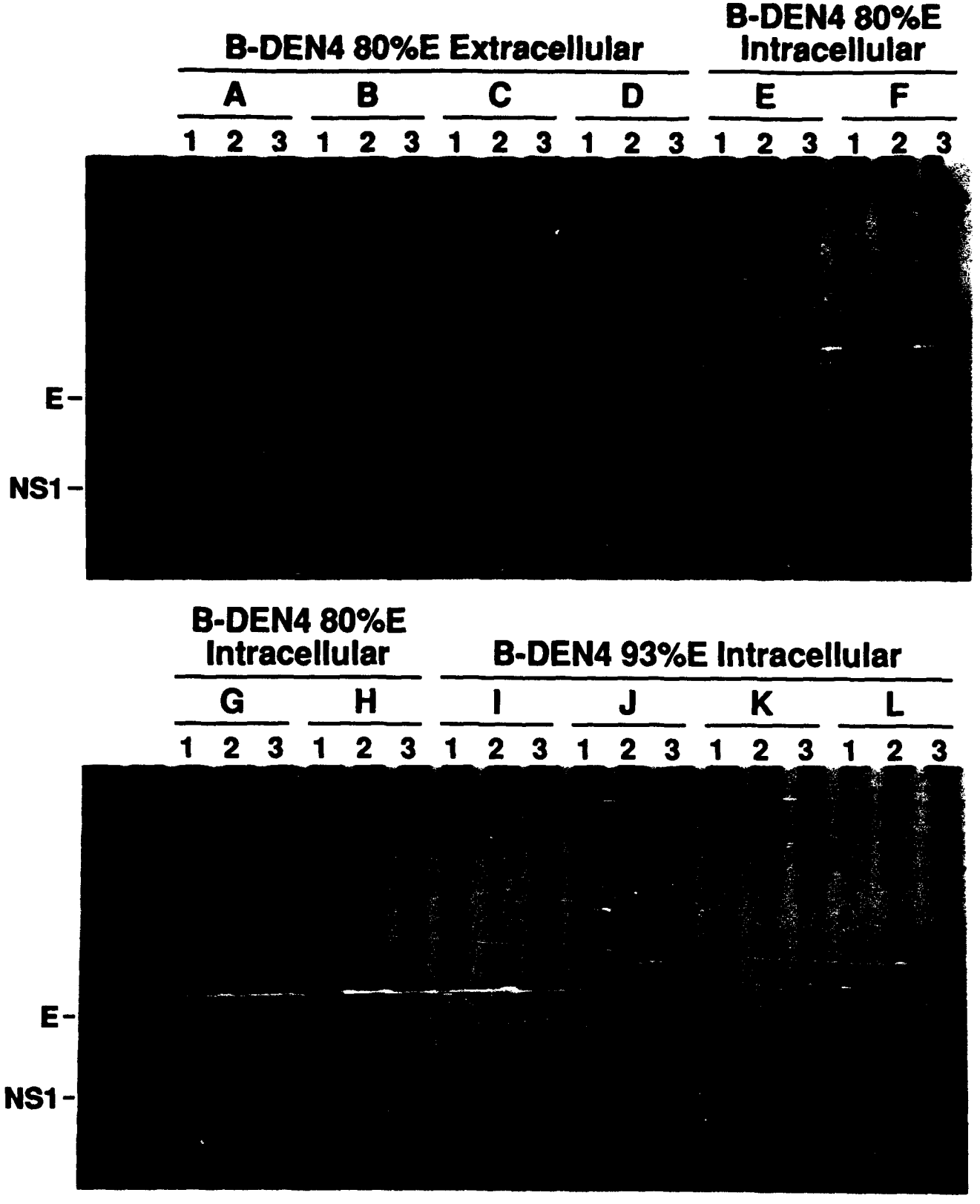

B-DEN4 80%E Extracellular
B-DEN4 80%E Intracellular
A
B
C
D
E
F
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
E-
NS1-
B-DEN4 80%E Intracellular
B-DEN4 93%E Intracellular
G
H
I
J
K
L
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
E-
NS1-

FIGURE 6. Detection of DEN4 E-specific antibodies in sera of immunized monkeys by radio-immunoprecipitation

Groups of four monkeys were immunized with recombinant baculovirus-expressed DEN 4 E proteins by intramuscular and subcutaneous routes: monkeys A, B, C, and D with concentrated medium of recombinant b(DEN 4, 80%E)-infected cells (80%E extracellular); monkeys E, F, G and H with a lysate of b(DEN4, 80%E)-infected cells (80%E intracellular); monkeys I, J, K and I with a lysate of b(DEN4, 93% E)-infected cells (93%E intracellular). Each monkey received a lysate of $1x10^7$ infected insect cells containing DEN4 93%E, 80% E, or a similar amount of the extracellularly secreted form of 80%E in 2 ml PBS/ dose. The monkeys were boosted with the same antigen preparation at 4 weeks and again at 15 weeks following the initial inoculation. Serum samples were collected 4 weeks after the initial inoculation (lane 1), 4 weeks after the first booster inoculation (lane 2), and 3 weeks after the last booster immunization (lane 3) from each monkey. DEN 4 E-specific antibodies were detected by radio-immunoprecipitation using ^{35}S-methionine-labeled proteins of DEN 4-infected C6/36 cells. The labeled precipitates were separated by SDS-PAGE. Molecular size markers (lane S) and the assigned DEN 4 protein bands as precipitated by HMAF (lane T) are shown.

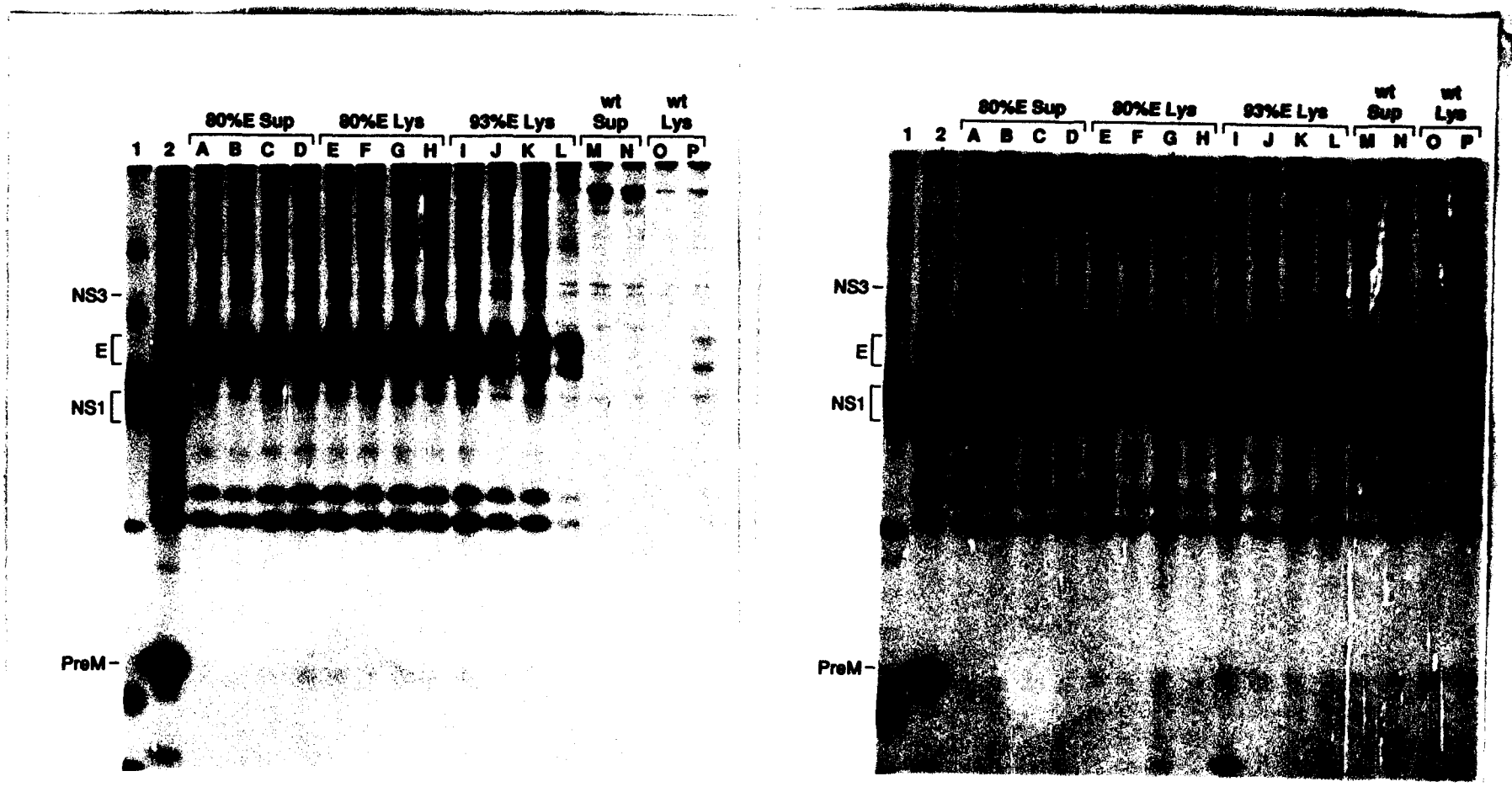


FIGURE 7. Seroresponse of immunized monkeys following dengue virus challenge

Serum samples collected at 2 weeks (left hand panel) and 6 weeks (right hand panel) post-challenge were analyzed by radio-immunoprecipitation using ^{35}S-methionine-labeled proteins of DEN4-infected C6/36 cells. The labeled immunoprecipitates were separated by SDS-PAGE. Molecular size markers (lane 1) and DEN4 protein bands precipitated by HMAF (lane 2) are shown.